Tall Fescue Doubled Haploids via Tissue Culture and Plant Regeneration[1]

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ABSTRACT

Genetic improvement of tall fescue (*Festuca arundinacea* Schreb.) by conventional breeding methodology is slow, and use of haploids and doubled haploids may accelerate the process. Androgenic haploid lines were evaluated in the field to identify differences in forage quantity and quality. Chromosome doubling to obtain fertility was attempted with some of the haploid lines. Direct regeneration of shoots from expanded ("aged") leaf midveins, as can be done with tobacco (*Nicotiana tabacum* L.), was not successful for tall fescue. However, calli from tall fescue were established in vitro from the lower end of rapidly elongating ("young") peduncles. Calli developed on such tissue explants within a few weeks on modified Linsmaier and Skoog medium with 2 mg of 2,4-D per L and no cytokinin. After 5 weeks, the calli were removed from the explants and subcultured on the same medium formulation at 24 ± 2°C for 15 weeks to "age" the callus tissue. The calli grew rapidly at first, but had stopped detectable growth and were beginning to senesce at 15 weeks. The "aged" calli were subdivided and placed on a plant regeneration medium (the same formulation but with only 0.25 mg of 2,4-D per L and no cytokinin). Leafy shoots began to emerge from the calli after 3 to 4 weeks of culture under continuous illumination from cool-white fluorescent lamps at about 15 μmol $m^{-2}s^{-1}$ in a room maintained at 23 ± 2°C. There were numerous green shoots and a few albinos. The green shoots were excised when they were about 1 cm tall and transferred to a rooting medium (devoid of both 2,4-D and cytokinin). The shoots developed roots within a few weeks and were transferred to sterile soil at high humidity. The humidity was decreased and light intensity increased over a several day period. After the plants were established, root tips were examined for somatic chromosome numbers. Of the normal-appearing plants, some had 21, others had 42, and a few had 81 to 84 chromosomes. Doubled haploids were developed by this method from six different haploid lines. One of the lines also produced a few quadrupled haploids. The regeneratd doubled and quadrupled haploids are being evaluated for forage characteristics and their potential usefulness to rapidly isolate desired characteristics.

***Additional index words:* *Festuca arundinacea*, Biotechnology, Plant cell culture, Anther culture, Aged callus, Doubling of chromosomes.**

GENETIC improvement of tall fescue (*Festuca arundinacea* Schreb.) by conventional breeding methodology is slow (11). Therefore, use of haploid and doubled haploid plants coupled with appropriate selection and evaluation schemes may be useful to speed up the process. Androgenic haploid plants offer a unique form of gametic selection; that is, plants with the gametic chromosome number can be evaluated under various environmental conditions to identify superior genotypes (1,3,8,18,19). Doubling the chromosomes of a haploid plant should produce a fertile line homozygous for the characteristics that were identified in the "parent" haploid plant (1,12). This procedure is potentially useful in forage grass improvement. However, methods for obtaining forage grass haploids and their doubled haploids need further development (7,10,15).

Although not directly applicable to tall fescue tissue culture, methodologies developed with species such as tobacco (*Nicotiana tabacum* L.) may serve as a model system. For example, numerous haploid tobacco plants can be cultured from immature anthers and screened to identify various disease resistances and plant characteristics (4,5,17). Midvein explants from the identified haploid tobacco plants can then be cultured on a regeneration medium to produce numerous leafy shoots from the explant. Regenerated shoots from expanding ("young") leaves of haploid tobacco plants are usually haploid, whereas those from fully-expanded ("aged") leaves of the same haploid plants include numerous doubled haploids. These doubled haploids are fertile and usually have the same physical and chemical characteristics identified in the "parent" haploid plant (16,17).

Tall fescue haploid plants have been cultured from immature anthers using an anther-panicle culture method (15). The haploid plants exhibited a range of physical characteristics, suggesting the possibility of identifying desired genotypes from among the haploid plants and doubling chromosome numbers via somatic tissue culture in a manner similar to that reported for tobacco (4,16). Unfortunately, direct regeneration of plants from fully-expanded leaf midvein explants was not successful in an earlier study with tall fescue (13). Apparently, a major difference between some of the forage grasses and tobacco is that only young, actively dividing tissue of the grasses develop callus (6,9,13), whereas callus initiates on both young and fully-expanded tobacco tissue. The objective of the present work was to develop doubled haploids from haploid tall fescue plants.

MATERIALS AND METHODS

Haploid Plants

Androgenic haploid plants were derived from anthers of field-grown tall fescue (*Festuca arundinacea* Schreb. 'Ky 31') by anther-panicle culture (15). Twenty-two cytologically verified haploids were increased through tillers. They were grown in pots in growth chambers with cool-white fluorescent lighting at about 600 μmol m^{-2} s^{-1} for 12 h per day and day/night temperatures of 20 ± 1°C. This combination resulted in rapid tillering. The tillering plants were subdivided several times to increase the material for replicated field evaluations and to provide material for chromosome doubling.

Selecting among Haploids

After increasing through tillers, the 22 haploid lines were grown in replicated field plots to evaluate forage quantity and quality under field conditions and to identify lines for chromosome doubling. There were 3 replicates, and each replicate contained 5 ramets of each of the 22 haploid lines

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(additional ramets of 6 of the lines were vernalized in another outdoor nursery the following year to provide material for chromosome doubling experiments). The ramets were spaced 30 cm apart in rows that were 90 cm apart. After over-wintering in the field, relative amounts of vegetative and reproductive growth were determined. The cold shock of winter did not result in any doubled haploid inflorescences. Vegetative growth was harvested, weighed (fresh weight), freeze-dried, weighed (dry weight), ground, and used to determine chemical components by conventional assays. Six of the lines with different physical markers were used for chromosome doubling. Presence of a marker (such as leaf angle, leaf shape, etc.) in both the haploid and its doubled haploid would be an indication of a successful doubling procedure.

Doubling Haploids

Five plants of each of the six selected haploid lines were brought from the outdoor vernalization nursery into a greenhouse in February 1981. Each plant was put in a separate pot. The greenhouse temperature was maintained at 20 ± 2°C. Supplemental lighting from cool-white fluorescent lamps provided 16-h daily photoperiods. Two plants of each haploid line were treated with colchicine on 2 Mar. 1981. Two more were treated on 10 Mar. 1981. The other plant served as an untreated control, and also provided tissue explants for the tissue culture approach to chromosome doubling. A second attempt with colchicine was made the following year.

Colchicine. The colchicine solution was 0.2% colchicine in distilled water. Treatment procedures were those used in grass breeding programs (2). After treatment, the plants were returned to the greenhouse. Daily observations for pollen shedding anthers were made after the panicles emerged. Panicles were harvested to determine whether seed developed.

Tissue culture. The untreated controls from the colchicine experiments were used to provide somatic tissue explants. When panicles were emerging (26 Mar. 1981), explants were taken from midveins of fully-expanded leaves. Other explants were taken from the lower ends of rapidly elongating peduncles. This "young" peduncle tissue contained many dividing and recently divided cells. The tissue explants were dipped into 70% (v/v) ethanol and then washed in 0.8% (v/v) sodium hypochlorite (15% Clorox[5] in water) for 6 min, followed by a wash in sterile distilled water. Surface decontamination of the tissue explants and transfer to culture media were done under a laminar flow transfer hood. Flame-sterilized instruments were used when appropriate. The media used to establish and maintain callus cultures and to regenerate plants were those described in a previous report (13). Two milligrams of 2,4-D was used per L of medium for callus establishment and "aging." The explants were cultured in disposable plastic petri dishes under continuous light (15 μmol m^{-2} s^{-1}) from cool-white fluorescent lamps at 23 ± 2°C. Calli soon initiated on the explants from young stem tissue, but not on those from the fully-expanded midveins. Calli were excised from the stem primary explants after 4 to 5 weeks. The calli were then placed on the same medium formulation and growth room conditions (without further subculturing) for about 15 weeks. The calli grew rapidly for about 4 weeks, then slowed and began to senesce by 15 weeks. The "aged" calli were then subdivided and placed on regeneration medium in disposable plastic petri dishes under the same light and temperature conditions discussed above.

For plant regeneration from the "aged" callus, we used 0.25 mg of 2,4-D per L and no cytokinin. Shoots regenerated from the calli were excised when they were about 1 cm tall, and they were rooted on medium without either auxin or cytokinin. The rooted shoots were transferred to sterile potting soil in 5-cm pots in plant growth chambers. Humidity surrounding the newly potted plantlets was maintained by covering them with a sheet of clear plastic. After a week, the plastic was slit to reduce the humidity around the plants. The opening was enlarged each day for another week, then removed entirely. The chambers were illuminated by cool-white fluorescent lamps for 12 h per day. Plants received about 600 μmol m^{-2} sec^{-1} and a temperature of about 21 ± 1°C. When the roots had elongated to about 2.5 cm, 1 to 2 cm root tips were removed and placed in distilled water at 4°C for approximately 20 h. The root tips were then placed in a saturated solution of mono-bromonaphthalene at room temperature for 3 h. After rinsing in distilled water, the roots were fixed in a 3 parts 95% ethanol: 1 part acetic acid (v/v) for 30 min. The root tips were rinsed again in water and stained in propiono-carmine for 12 h. Lastly, the roots were placed in 6 *N* HCl for 10 min and then stored in distilled water at 4°C. The root tips were squashed in propionocarmine for observation under a light microscope.

Doubled haploid plants were increased through tillers, as described above, and grown in greenhouses and outdoor nurseries. Representative plants were photographed to document results.

RESULTS AND DISCUSSION

Screening Haploid Plants

Cytologically verified haploid plants, which were previously derived by anther-panicle culture (15) and then increased via tillers, displayed differences in phenotypic and forage quality traits when grown under field conditions. Relative amounts of vegetative and reproductive growth among the various lines were determined after overwintering in the field (Table 1). The lines ranged from those that produced few panicles and many leaves to those that produced many panicles and few leaves. Chemical analyses of the forage also indicated differences among the various haploid lines (14). For example, sugar, neutral detergent fiber and moisture ranged from 5.0 to 7.8%, 48 to 59%, and 63 to 72%, respectively, for summer harvest of field-grown samples. The important point is that differences existed among the haploid lines, while field growth from the 15 ramets was very uniform within each haploid line.

Differences for the measured traits among the various haploid lines support the hypothesis that the lines originated from different microspores, and are genetically different. Thus, it is possible to identify different genotypes among tall fesuce haploid plants, as was done with tobacco haploids (4,5,17). Repeated cloning via tillers demonstrated high stability of the haploids and provided material for various plant evaluations. No spontaneously doubled haploids were detected among the tiller-propagated plants of any of

[5] Mention of a trade name does not constitute a guarantee or warranty of the product by the USDA and does not imply approval to the exclusion of other products that may also be suitable.

Table 1. Relative amounts of vegetative and reproductive growth of the different androgenic haploid lines (15) under field conditions.

Haploid line	Field growth			
	Veget.	Reprod.	Dry matter	
	22 July		25 Aug.	28 Oct.
	rating†		g dry wt/5 plants	
1‡	8	6	54	100
3	2	10	15	27
4	6	6	41	55
5	10	3	55	91
6	8	4	59	77
7	8	1	51	75
8	8	4	52	84
9	9	4	52	92
10	9	4	59	101
11	10	4	76	104
12	6	6	26	44
13	2	4	7	7
14	9	3	58	134
15	10	3	60	111
16	10	4	58	132
17	4	6	18	31
18	10	5	69	126
19	8	6	66	111
20	10	4	59	111
21	1	10	–	–
22	9	6	71	103
23	8	5	52	92
LSD 0.05			16	32

† Rating scale is 1 to 10. 1 = least, 10 = most.
‡ Haploid line numbers are those used in the paper by Kasperbauer et al. (15). Plant No. 2 was not a haploid, therefore, it is deleted from this table.

the haploid lines. Therefore, we then concentrated on procedures to develop doubled haploids in order that the materials could be of more practical use in tall fescue improvement. Haploids that were overwintered in the field were used for cohchicine treatments, and untreated plants provided a source of somatic tissue for the tissue culture approach to chromosome doubling.

Doubling Haploids

Colchicine. No doubled haploids resulted from colchicine treatment in either year. That is, no pollen was shed and no seed developed on any of the treated plants. It is possible, however, that greenhouse conditions influenced pollen development and that lack of seed set may have been due, in part, to the limited amount of pollen available from other tall fescue plants in the greenhouse. However, even if the colchicine-treated plants had set seed, it is highly probable that the seed would have been hybrid because tall fescue is wind-pollinated and highly self-sterile. Thus, an alternate doubling method was desired. Our goal was to develop procedures to obtain doubled haploid plants that could be cytotogically-verified, vegetatively increased in number and evaluated under field or other stress conditions before introducing them into a breeding program. The earlier experience with tobacco suggested that tissue culture-derived doubled haploids might be suitable for this purpose.

Tissue culture. Our first approach was to attempt with tall fescue what was successful with tobacco; i.e., direct regeneration of doubled haploid shoots from excised midveins of fully-expanded leaves from cytologically-verified haploid plants (16). However, direct regeneration of leafy shoots from excised fully-expanded midveins of tall fescue was not successful. This was consistent with our earlier findings with annual ryegrass (*Lolium multiflorum* Lam.) × tall fescue hybrids (13). Both the present and previous findings were in contrast to tobacco (and some other dicots) in which numerous leafy shoots could be regenerated directly from excised midveins of fully-expanded leaves (16). Consequently, another approach was needed for tall fescue.

Table 2. Somatic chromosome numbers in plants regenerated from 'aged' somatic-derived callus tissue from cytologically-verified (21 chromosome) haploid plants of tall fescue.

Haploid source plant	No. of regenerated plants	No. of regenerated plants with indicated somatic chromosome numbers					
		21	38	40	41	42	81–84
No. 6	7	6				1	
No. 11	6	5				1	
No. 14	7	5		1		1	
No. 15	19	8		2	1	8	
No. 18	4	2	1			1	
No. 20	17	11				2	4
Total	60	37	1	3	1	14	4

In the earlier forage grass work (13), we found that "young" somatic tissue with numerous dividing or recently divided cells was capable of forming callus in vitro on an appropriate nutrient medium with about 2 mg of 2,4-D per L. Also, the callus could be subcultured. Shoots could be regenerated from the calli by decreasing the 2,4-D content of the medium. In that work we found that plants regenerated from callus after one subculture had fewer aneuploids and somaclonal variants than did plants regenerated after numerous subcultures. Thus, our goal was to regenerate doubled haploid plants from haploid-derived callus after one subculture.

Given the previous success with "aged" leaf midveins of tobacco and the consistent failure to establish callus or regenerate plants from "aged" midveins of tall fescue, we hypothesized that it may be possible to regenerate doubled haploid tall fescue plants from callus that was established from "young" somatic tissue, subcultured once and then "aged" as callus before regenerating plants from the callus. Details of the procedures used for regeneration of doubled haploid shoots from somatic tissue excised from tall fescue haploids relative to procedures used for regeneration of doubled haploids of tobacco are shown in Fig. 1. Somatic chromosome numbers in the plants regenerated from the "aged" tall fescue calli are shown in Table 2. In other experiments, higher percentages of plants with increased chromosome numbers were regenerated from calli that had undergone numerous subcultures before regenerating plants. However, many of the regenerated plants were aneuploid and/or somaclonal variants. This was undesirable in the present effort because the goal was to obtain doubled haploids with characteristics identified in the "parent" haploid plant.

Tissue culture can be used to obtain doubled haploid and even a few quadrupled haploid plants from

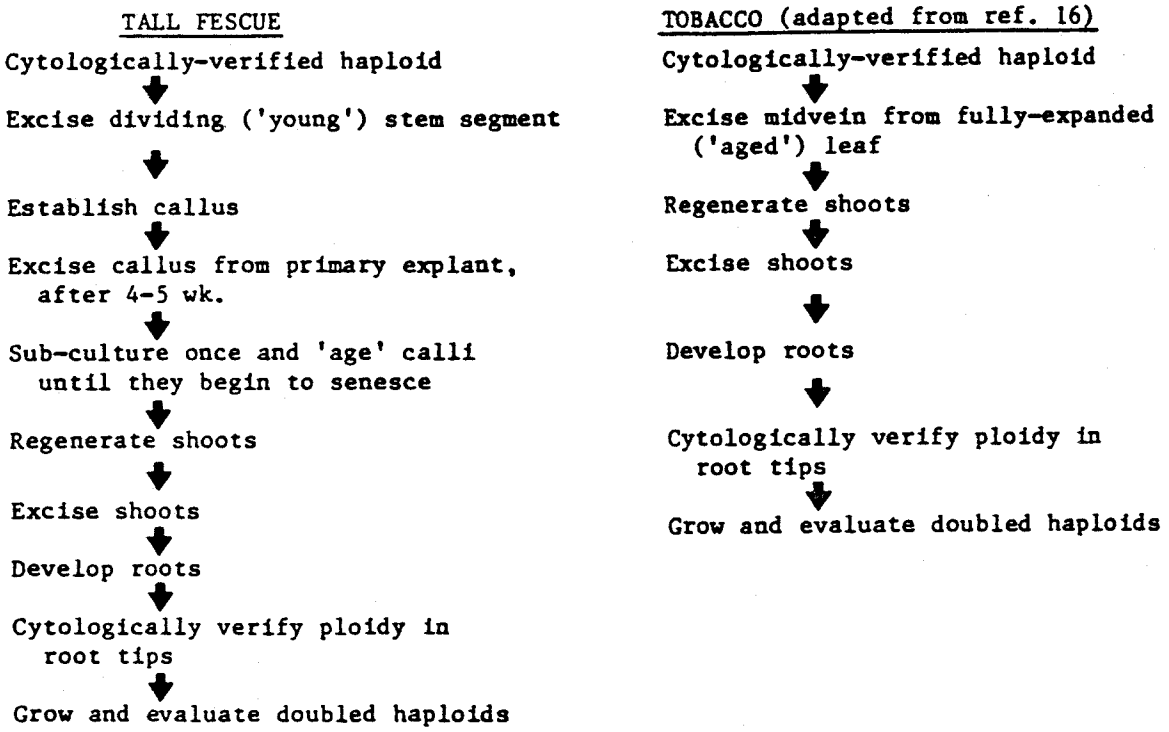

Fig. 1. Flow chart comparing the regeneration of doubled haploid shoots from somatic tissue excised from tall fescue haploids with procedures used for tobacco.

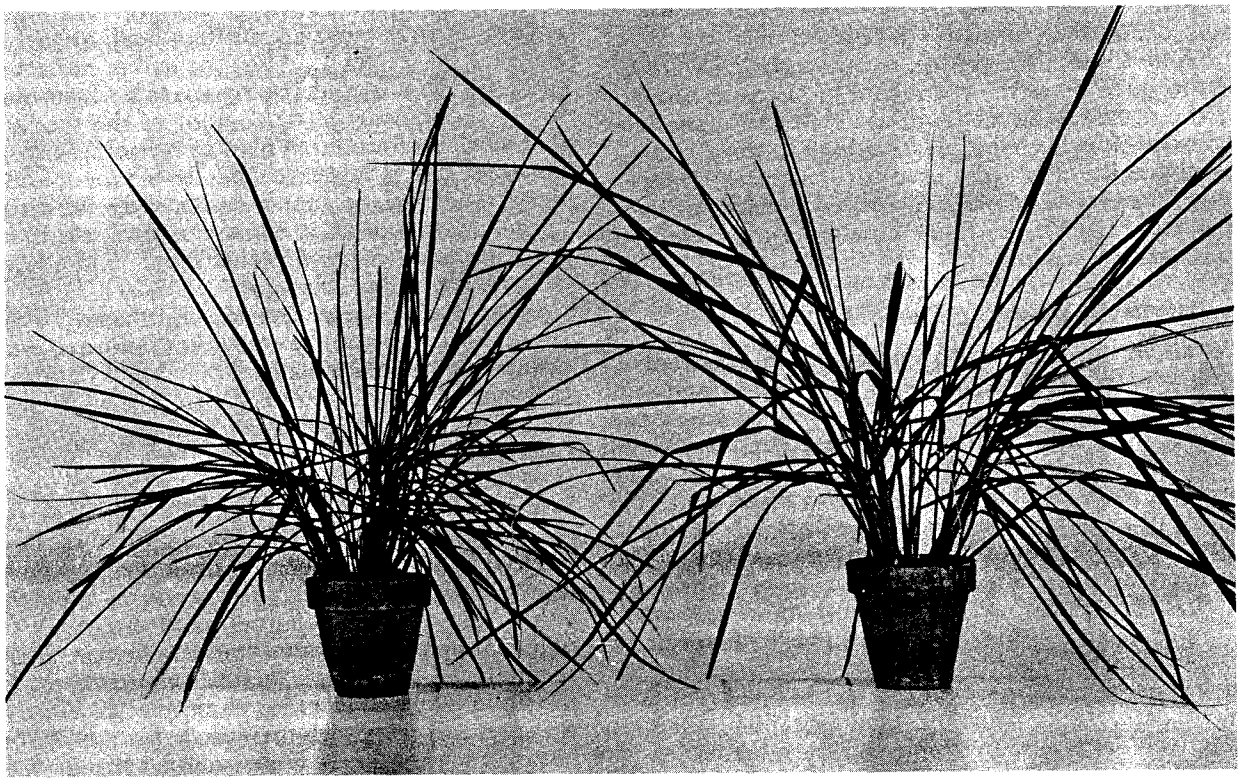

Fig. 2. An androgenic haploid (left) derived via anther-panicle culture (15) and a doubled haploid derived via 'aged' somatic callus culture from the haploid (right).

cytologically-verified tall fescue haploids. The fact that one or more doubled haploid plants were regenerated from each of the six different haploid lines suggests that the tissue culture approach is valid for doubling tall fescue haploids.

A representative haploid plant and one of the tissue culture derived doubled haploids are shown in Fig. 2. Both plants shown in the figure were started at the same time from tillers of the respective ploidies. The plants in Fig. 2 appear to be similar in leaf shape and

leaf angle. However, as expected, the doubled haploid plant parts are about 25% larger. This is similar to observations of haploid and doubled haploid tobacco plants in which the doubled haploid plants have leaves, flowers, and stems that are about 25 to 30% larger than comparable components of the haploids (16).

It is apparent that haploid plants can be cultured from tall fescue (15). Increasing the haploids through tillers resulted in the propagation of stable haploid plants and allowed field evaluation for forage quantity and quality factors. After screening under field conditions, ploidy of selected haploid plants can be increased through tissue culture. Use of tissue culture to obtain haploid and doubled haploid tall fescue offers the possibility of more rapid identification of superior genotypes among the haploids and then culturing fertile doubled haploids with the characteristics identified in the haploids. An alternate procedure is to double chromosome numbers of all haploids, increase them via tillers, and then compare the doubled haploid lines with the best available cultivars as a check in field plots. Both procedures have merit and depend on availability of haploids, derivation of stable doubled haploids, and space to evaluate them. The present study concentrated on methodology for development of doubled haploids from previously-derived androgenic haploids. Additional studies are in progress on chromosome pairing in the doubled haploids and on cross-fertility of the doubled haploids with conventionally derived breeding lines.

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